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Positive Correlation Between Somatic Mutations in *RAS* Gene and Colorectal Cancer in Telangana Population: Hospital-Based Study in a Cosmopolitan City

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Abstract

Colorectal cancer (CRC) ranks among the most prevalent cancer types in both men and women. Screening of *RAS* (Kirsten rat sarcoma viral oncogene homolog (*KRAS*), neuroblastoma RAS viral oncogene homolog (*NRAS*), and v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*)) somatic mutations is necessary prior to considering anti-epidermal growth factor receptor (EGFR) therapies in CRC patients. Next-generation sequencing studies have confirmed that *RAS* gene panels could be used while developing treatment strategies for patients with CRC. The present study explored genetic mutations in *KRAS*, *NRAS*, and *BRAF* in CRC patients in the Telangana state of India. Patients with confirmed CRC (n = 100) who visited the Apollo hospitals were evaluated. Genomic DNA was extracted from formalin-fixed, paraffin-embedded tissues, and pyrosequencing analysis was performed. Patient DNA samples were screened for 54 different *KRAS*, *NRAS*, and *BRAF* mutations, which revealed 34 somatic mutations. Exon 11 of *BRAF* possessed 4 mutations with highest individuals documented with G469A mutation. Pyrosequencing, a reliable method for analyzing somatic mutations present in *RAS*, could aid in taking treatment decisions for patients with CRC.

Keywords Colorectal cancer · *RAS* · *KRAS* · *NRAS* · *BRAF*

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Introduction

Cancer is the second most common multifactorial disease that leads to death worldwide [2], with colorectal cancer (CRC, OMIM #114500) being the fourth leading cause of cancer-related deaths globally [17, 19]. Based on epidemiological investigations, most CRC cases could be attributed to environmental factors [18, 28]. Genetic evidences have revealed that individuals with specific single nucleotide polymorphisms (SNPs) might be more cancer susceptible [11]. Somatic mutations in Kirsten rat sarcoma viral oncogene homolog (*KRAS*), neuro-blastoma RAS viral oncogene homolog (*NRAS*), and v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) genes have been confirmed in routine screening of patients with advanced-stage CRC by next-generation sequencing (NGS) [6]. In humans, 3 RAS gene mutation occurs in 90% of the pancreatic cancers, followed by colon (45%) and lung cancers (35%), respectively. *RAS* is a common target in oncology-related drug development [26], and its mutations act as important biomarkers for predicting the response to anti-epidermal growth factor receptor (EGFR) antibody-based therapy in CRC [3]. For CRC development and progression, EGFR signals play a major role [10]. Three RAS genes (*KRAS*, *NRAS*, and *BRAF*) code for kinases involved in the RAS-RAF-mitogen-activated protein kinase signaling pathway and their mutations lead to the resistance against anti-EGFR monoclonal antibody therapy. Most of the *KRAS* mutations (98.5%) occur at codons 12 and 13 of exon 2. *NRAS* mutations typically occur in exon 2-4. In *BRAF*, nearly all (81.9%) mutations occur in codon 600 (V600E) [32]. Only a few studies have included Indian populations for analyzing all the *RAS* (*KRAS-NRAS-BRAF*) mutations in CRC cases [1, 12]. Therefore, this study was conducted to assess the association of *KRAS*, *NRAS*, and *BRAF* somatic mutations with CRC risk in the population of Telangana, India.

Subjects and Methods

Patient Selection

In this study, patients who had been diagnosed with CRC (n = 100) between January, 2014, and December, 2018, at the Department of Oncology, Apollo Hospitals, Jubilee Hills, Hyderabad, Telangana, India, were enrolled. The inclusion criteria were a confirmed diagnosis, based on histopathological examinations and primary CRC. Exclusion criteria included the presence of any other cancers, prior anti-cancer treatment, and non-adenocarcinomas of the colon. This study was conducted at the Department of Molecular Biology and Cytogenetics, Apollo Hospitals, Hyderabad, India.

DNA Isolation

CRC biopsy samples (100), placed in 10% buffer for formalin-fixed paraffin-embedded (FFPE) tissue(s) preparation were enrolled. DNA from FFPE tissues was extracted using Qiagen kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. DNA quality based on concentration and purity was assessed with a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Purified DNA was used for the pyrosequencing analysis.

Pyrosequencing Analysis

In this study, polymerase chain reaction (PCR) was performed with a pyrosequencing method using specific primers designed to detect *KRAS*, *NRAS*, and *BRAF* mutations. The target sequence covering the polymorphic site was amplified with one of the specific biotinylated primers. Pyrosequencing analysis was carried out as described previously [4, 24]. The total PCR volume and thermal cycler conditions were set in accordance with those previously described by Khan et al. [14], except for the annealing temperatures, which were modified. The primers, master-mix, and protocol were supplied by Qiagen. Table 1 provides a list of the tested mutations during screening.

Results

In this study, 100 CRC cases were screened and the clinicopathological characteristics of tumors or FFPE tissues (obtained from the patients) were evaluated. Among the CRC cases studies, 63% were men, whereas, 37% were women, with a mean patient age of 59.1 ± 11.92 years. It was noted that 33% of patients were smokers, while 28% consumed alcohol. All colon tissue samples were > 5 cm in size. Family histories were collected for only 22% of the CRC cases. Specific clinical pathological stages were not documented for all the patients, and 67% of cases were stage II (67/100). The baseline characteristics are given in Table 2. Pyrosequencing analysis was carried out to detect *KRAS*, *BRAF*, and *NRAS* mutations in patients with CRC. Overall, 13% of the patients possessed mutations in *KRAS*. Exon 2 of *KRAS* exhibited G12C (3%), G13D (2%), G12A (3%), G12D (2%), and G12S (1%) mutations, whereas, Q16L (1%) and Q16E (1%) mutations were detected in exon 3 of *KRAS* (Table 3). Fifteen (15%) patients exhibited mutations in exon 11 of *BRAF*, with G1397T (2%), G1406C (1%), G1391A (3%), G1405T (1%), G1406C (4%), G1406C (4%) (maximum), and G1391T (2%) mutations.

Table 1 List of the mutations present in the exons, codons, and specific *RAS* genes in CRC patients

S. no	Gene	Codon	Mutations	Exons
1	*KRAS*	Codon 12	G12A, G12C, G12D, G12R, G12S, G12V	Exon-2
2	*KRAS*	Codon 13	G13D	Exon-2
3	*KRAS*	Codon 59	A59T, A59G	Exon-3
4	*KRAS*	Codon 61	G12A, G12C, G12D, G12R, G12S, G12V	Exon-3
5	*KRAS*	Codon 117	K117	Exon-4
6	*KRAS*	Codon 146	A146T, A146P, A146V	Exon-4
7	*NRAS*	Codon 12	G12S, G12C, G12R, G12D, G12V, G12A	Exon-2
8	*NRAS*	Codon 13	G13S, G13C, G13R, G13D, G13V, G13A	Exon-2
9	*NRAS*	Codon 59	A59T, A59G	Exon-3
10	*NRAS*	Codon 61	Q61K, Q61R, Q61L, Q61H, Q61H, Q61Q	Exon-3
11	*NRAS*	Codon 117	K117N	Exon-4
12	*NRAS*	Codon 146	A146T, A146P, A146V	Exon-4
13	*BRAF*	Codon 464	G464E, G464V	Exon-11
14	*BRAF*	Codon 465	None	Exon-11
15	*BRAF*	Codon 466	G466E, G466V	Exon-11
16	*BRAF*	Codon 467	None	Exon-11
17	*BRAF*	Codon 468	None	Exon-11
18	*BRAF*	Codon 469	G469A, G469E, G469V	Exon-11
19	*BRAF*	Codon 600	V600A, V600E, V600G, & V600M	Exon-15

Table 2 Demographic characteristic details of both CRC patients and control subjects

Clinical characteristics	CRC (n = 100)
Age (years)	59.1 ± 11.92
Gender (male/female)	(63:37)
Smoking	33 (33%)
Drinking	28 (28%)
Tumor location (rectum/colon)	0 (0%)/100 (100%)
Tumor size (< 5 or > 5 cm)	0(0%)/100 (100%)
Clinical pathological (stage I)	NA
Clinical pathological (stage II)	67 (67%)
Clinical pathological (stage III)	NA
Clinical pathological (stage IV)	NA
Family history of CRC	22%

NA not applicable

In exon 15 of *BRAF*, G1798A (1%) and T1799A (1%) mutations were detected. In addition to this, 3 patients exhibited mutations in exon 3 of *NRAS* (G175A (2%) and A182T (1%) mutations) (Table 3; Fig. 1).

Discussion

Molecular testing has become more important in cancer-effected patients, due to the improvements in targeted therapy [16]. Well-documented genetic mutations in *RAS* (*KRAS*, *NRAS*, and *BRAF*) genes aid in evaluating patients with CRC and have a high rate of incidence and mortality, which is lowering because of the recent advancements in disease identification through genetic screening, and is useful in making treatment decisions. Biologically and epidemiologically, CRC is a heterogenous disease with multiple etiologies. This heterogeneity impacts its prognosis and clinical management, as the tumor tissue's location does not accurately reflect *KRAS* mutation's status in the entire tumor [20]. In 100 patients with

Table 3 Clinical analysis of the pyrosequencing results in CRC cases and healthy controls

Gene	Exon	Mutation	Amino acid	Nucleotide	CRC cases (n = 100)
KRAS	Exon-2	GGT>TGT	G12C	G>T	03 (3%)
KRAS	Exon-2	GGC>GAC	G13D	G>A	02 (2%)
KRAS	Exon-2	GTT>GCT	G12A	T>C	03 (3%)
KRAS	Exon-2	GGT>GAT	G12D	G>A	02 (2%)
KRAS	Exon-2	GGT>AGT	G12S	G>A	01 (1%)
KRAS	Exon-3	CAA>CTA	Q61L	A>T	01 (1%)
KRAS	Exon-3	CAA>GAA	Q61E	C>G	01 (1%)
BRAF	Exon-15	1799 T>A	V600E	T>A	01 (1%)
BRAF	Exon-15	1798-1799 GT>AA	V600K	GT>AA	01 (1%)
BRAF	Exon-11	1397 G>T	G466V	G>T	02 (2%)
BRAF	Exon-11	1406 G>C	G469A	G>C	01 (1%)
BRAF	Exon-11	1391 G>A	G464E	G>A	03 (3%)
BRAF	Exon-11	1405-1406 GG>TC	G469S	GG>TC	01 (1%)
BRAF	Exon-11	1406 G>C	G469A	G>C	04 (4%)
BRAF	Exon-11	1391 G>T	G464V	G>T	02 (2%)
NRAS	Exon-3	175 G>A	A59T	G>A	02 (2%)
NRAS	Exon-3	182 A>T	Q61L	A>T	01 (1%)

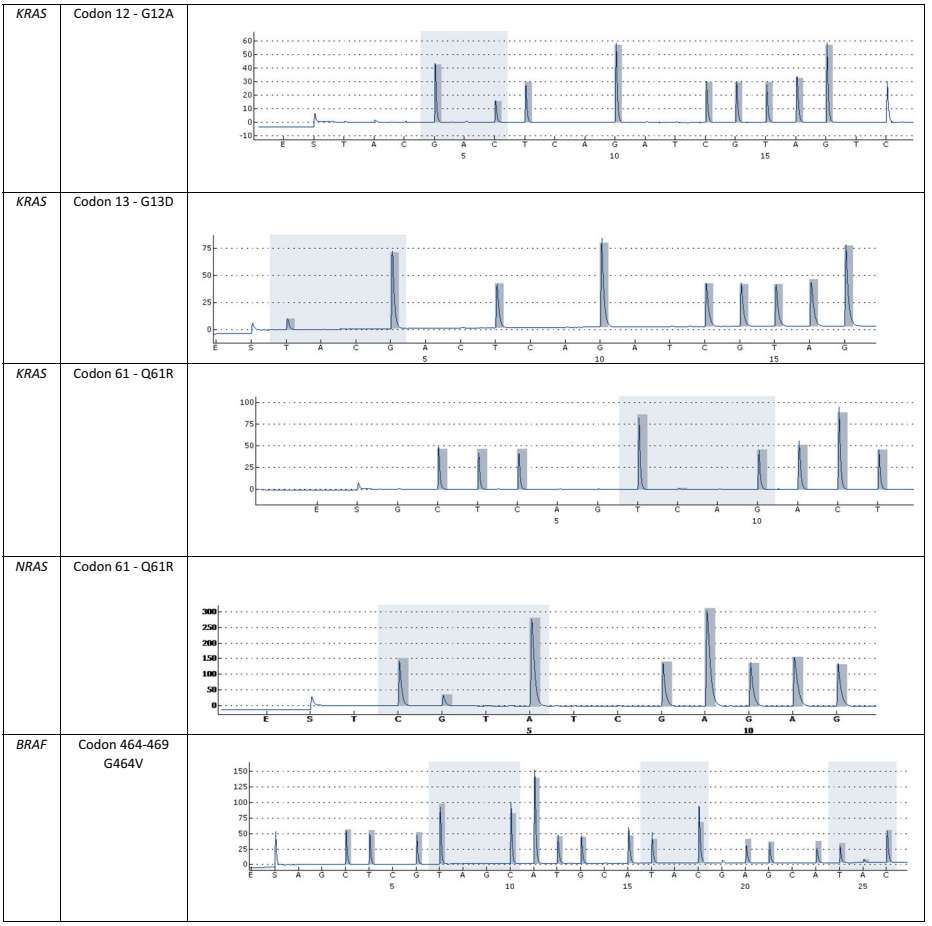

Fig. 1 Reference pictures for pyrosequencing analysis

CRC, 54 mutations were screened and 31 were detected in *KRAS*, *NRAS*, and *BRAF*. In *KRAS*, *NRAS*, and *BRAF*; 13, 38, and 3% of the mutations were detected, respectively.

The RAS gene family is well documented to be cancer associated with mutations in codons 12 and 13 of *KRAS*, observed in 35–45% of all CRC cases. Detecting *KRAS* mutational status has become a crucial tool for managing the patients with CRC [5]. These mutations are recognized as biomarkers for predicting stage IV CRC and indicate poor efficiency of anti-EGFR therapy. However, *KRAS* mutation's prognostic value remains controversial, with some studies on patients with stage II and III CRC, revealing a positive association [15]. The subjects evaluated in the current study were mainly in stage II CRC. In mammalian cells, *RAS* gene encodes monomeric GTPases that are involved in signal transduction pathways and regulate cell proliferation and differentiation [31]. *RAS* gene has primarily been documented as a viral gene, and its mutations are detected in 30% of the human cancer cells. KRAS protein (also known as p21), a member of the RAS superfamily of proteins, is a membrane-anchored GTP/GDP-binding protein that is broadly expressed in human cells [29]. The use of monoclonal antibodies targeting EGFR (cetuximab and panitumumab) as combination chemotherapy has exhibited clinical benefits in patients, with a response rate of ~ 20% for metastatic

CRC [27]. *KRAS* is a proto-oncogene, mainly involved in the RAS-MAPK signaling pathway to regulate cell growth, proliferation, and differentiation. Mutations frequently occur around KRAS gene codons, converting normal cells into cancerous. Whole genome sequencing studies confirmed that *KRAS* mutations are triggered in limited number of carcinomas, with CRC being one of them and *KRAS* mutations indicate a residual tumor [7]. KRAS was initially identified as a p21GTPase, and a high rate of somatic mutations in *KRAS* was observed in carcinomas such as leukemia, non-small cell lung cancer, pancreatic cancer, and CRC. Most of these mutations were present in the exons 2 and 3, with the common mutations including G12C, G12D, G12R, G12S, G12V, G13D, and Q16H, whereas, exon 4 displayed K117 and A146G mutations [31]. In our study, G12C, G13D, G12A, G12D, G12S, Q61L, and Q61E mutations in *KRAS* were detected. In general, *KRAS* mutational analysis is performed by Sanger sequencing analysis or pyrosequencing. Pyrosequencing technique is considered as a homogenous genotype that concerns *KRAS* mutation status in tumor cells. When *KRAS* mutations are left undetected by DNA sequencing, anti-EGFR therapy may fail to have antiproliferation effects; therefore, performing pyrosequencing analysis with KRAS mutation kit (Qiagen) is recommended. Codons 12 and 13 in exon 2 of *KRAS* alter the protein sequence. Changes in the first 2 base pairs result in amino acid substitution in KRAS protein, resulting in the treatment [34].

The frequency of other KRAS mutations at codons 16, 146, and 154 were found to be approximately 5%. However, among the somatic mutations in cancer database, 500 mutations (approximately) have been detected in *KRAS* gene in CRC samples [29]. Mutations in *RAS* genes such as *KRAS* and *NRAS* (in exons 2-4) indicate that these metastatic CRC patients might be resistant to anti-EGFR targeted therapy [13]. *BRAF* mutations are potential prognostic markers of CRC therapy. At codon 600, the conversion of amino acid valine to glutamic acid (V600E) is responsible for CRC development in 10% of the cases. A correlation between the combination of *KRAS* and *BRAF* mutations and the prognosis has been demonstrated and might be useful for developing cancer treatments [30].

The prevalence of *KRAS*, *NRAS*, and *BRAF* mutations have been evaluated in global studies; however, limited data is available regarding their respective frequencies in CRC [8–10, 21–23, 25, 33]. Results of the present study were not in agreement with that of other studies [8–10, 22, 25, 33], which might be due to ethnicity, modified lifestyle, and/or genetics (Table 4).

Pyrosequencing analysis was performed to detect the mutations in *RAS* genes in patients with CRC. We have predicted that CRC patients with *KRAS* mutations might not benefit from anti-EGFR therapy, which however, might treat metastatic CRC and the patients should be screened for *KRAS* mutations before therapy administration. Furthermore, the study also lacked control tissues and subjects, thereby, making the information of the clinical pathological

Table 4 Comparison of the study results with other ethnic populations

Authors	*KRAS*	*NRAS*	*BRAF*
Current study	13	38	3
Hamzezadeh 2018	28.7%	0%	Not analyzed
Gilson et al. 2019	17/38	20/38	20/38
Guedes et al. 2013	44.1%	Not analyzed	18.6%
Suhaimi et al. 2015	31.8%	11.3%	Not analyzed
Payandeh et al. 2015	36.4%	0%	0%
Zhang et al. 2015	45.6%	3.9%	3.1%

stages incomplete. Additionally, studies examining large number of patients are required. The presence of somatic mutations in *KRAS*, *NRAS*, and *BRAF* genes has been confirmed in the routine screening of patients with advanced-stage CRC, with NGS [6].

Limited studies including RT-PCR, NGS, and Sanger sequencing, to rule out the disease, have been previously performed; however, in the present study, pyrosequencing was performed to detect CRC-associated *RAS* mutations. In this study, including 100 samples is an advantage which constitutes a high sample number when comparing with other ethnic population(s). The similarity of the current study with other global studies is the screening of similar mutations in *RAS* genes.

In conclusion, we confirmed and suggested *RAS* genes screening by pyrosequencing prior to anti-EGFR therapy administration in patients with CRC. Pyrosequencing analysis of the *RAS* genes revealed high clinical sensitivity, which could be used for determining the effective treatments for patients with CRC. Pyrosequencing could be performed by the pathologists/molecular biologists in order to obtain clinical confirmation that further aid in developing strategies for treating cancer.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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